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### Title

Effects of single parenthood on mothers' behavior, morphology, and endocrine function in the biparental California mouse.

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## **Highlights**

1. Morphological, endocrine and behavioral effects of single motherhood on mothers are not well known.
2. We evaluated such effects in the monogamous, biparental California mouse.
3. Single mothers (SM) spent more time engaged in maternal behavior than paired mothers (PM).
4. SM had heavier livers and hearts, but smaller ovaries and uteri, than PM and non-breeding females.
5. SM had a flatter diel CORT rhythm and a smaller CORT stress response than non-breeding females.

Effects of single parenthood on mothers' behavior, morphology, and endocrine function in the  
biparental California mouse

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## Abstract

Motherhood is energetically costly for mammals and is associated with pronounced changes in mothers' physiology, morphology and behavior. In ~5% of mammals, fathers assist their mates with rearing offspring and can enhance offspring survival and development. Although these beneficial consequences of paternal care can be mediated by direct effects on offspring, they might also be mediated indirectly, through beneficial effects on mothers. We tested the hypothesis that fathers in the monogamous, biparental California mouse (*Peromyscus californicus*) reduce the burden of parental care on their mates, and therefore, that females rearing offspring with and without assistance from their mates will show differences in endocrinology, morphology and behavior, as well as in the survival and development of their pups. We found that pups' survival and development in the lab did not differ between those raised by a single mother and those reared by both mother and father. Single mothers spent more time in feeding behaviors than paired mothers. Both single and paired mothers had higher lean mass and/or lower fat mass and showed more anxiety-like behavior in open-field tests and tail-suspension tests, compared to non-breeding females. Single mothers had higher body-mass-corrected liver and heart masses, but lower ovarian and uterine masses, than paired mothers and/or non-breeding females. Mass of the gastrointestinal tract did not differ between single and paired mothers, but single mothers had heavier gastrointestinal tract compared to non-breeding females. Single motherhood also induced a flattened diel corticosterone rhythm and a blunted corticosterone response to stress, compared to non-breeding conditions. These findings suggest that the absence of a mate induces morphological and endocrine changes in mothers, which might result from increased energetic demands of pup care and could potentially help maintain normal survival and development of pups.

24 Keywords: single mothers; maternal care; biparental care; morphology; corticosterone

25

26 **Highlights:**

27 1. Metabolic and behavioral effects of single motherhood on mothers have rarely been explored.

28 2. We tested for such effects in the monogamous, biparental California mouse.

29 3. Single mothers spent more time in feeding behaviors than paired mothers.

30 4. Single mothers had higher liver and heart masses, but lower ovarian and uterine masses, than  
31 both paired mothers and non-breeding females.

32 5. Single motherhood induced a flattened diel corticosterone rhythm and a blunted  
33 corticosterone response to stress, compared to non-breeding conditions.

## 34 Introduction

35 Maternal care is costly for female mammals and is associated with pronounced morphological,  
36 physiological, and behavioral changes in mothers (Lonstein, 2007; Slattery and Neumann, 2008;  
37 Speakman, 2008). In small mammals, for example, mothers often undergo declines in fat stores  
38 during lactation. They can also experience organ remodeling, such as growth of the alimentary  
39 tract and associated organs such as the liver and pancreas, which may be necessary to meet the  
40 high demands of lactation (Jolicoeur et al., 1980; Kennedy et al., 1958). Consequences of the  
41 costs of motherhood include reductions in thermogenesis and physical activity, loss of bone mass,  
42 and disruption of sleep patterns (Speakman, 2008).

43 Mothers in some mammalian species also exhibit blunted hormonal, neural and  
44 behavioral responses to stressors during late pregnancy and lactation (Brunton et al., 2008;  
45 Lightman et al., 2001; Slattery and Neumann, 2008). Furthermore, pregnant and lactating rats,  
46 mice and possibly humans exhibit reduced anxiety and fearfulness. After becoming mothers, for  
47 example, rats (*Rattus norvegicus*) and house mice (*Mus spp.*) have reduced acoustic startle  
48 responses, increased locomotion in open-field tests, increased time spent in the open arms of the  
49 elevated plus maze and reduced fleeing from an intruder (Lonstein, 2007; Lonstein et al., 2014;  
50 Slattery and Neumann, 2008).

51 Although most mammals are uniparental, with only mothers caring for their offspring,  
52 approximately 5% are biparental, with fathers helping to rear their young (Kleiman and Malcolm,  
53 1981). Depending on the species, paternal care in mammals can include a variety of behaviors,  
54 including retrieving, defending, playing with, grooming and huddling offspring, as well as  
55 providing them with food and a nest (Kleiman and Malcolm, 1981). In some species, such as the  
56 California mouse (*Peromyscus californicus*), fathers spend as much time interacting with their

offspring as do mothers (Bester-Meredith et al., 1999; Woodroffe and Vincent, 1994). In other species, such as prairie voles (*Microtus ochrogaster*), fathers' paternal behavior may compensate for low levels of maternal care (Perkeybile et al., 2013): offspring receive more care from fathers when mothers display low care. Therefore, fathers in biparental species can reduce the energetic burden of motherhood.

Fathers in biparental species can also influence offspring survival and development. For example, in California mice, paternal care has been found to greatly enhance survival and development of pups both in the field and under energetically challenging lab conditions, such as low ambient temperature or having to work for food (Bredy et al., 2007; Cantoni and Brown, 1997; Dudley, 1974; Gubernick and Teferi, 2000; Gubernick et al., 1993; Wright and Brown, 2002). The absence of a father can also influence offspring's cognitive, emotional, and reproductive behavior. In California mice, permanent removal of the father on postnatal day 3 decreases spatial learning ability (Bredy et al., 2004), and in mandarin (*M. mandarinus*) and prairie voles, paternally deprived offspring are more anxious and less social on multiple measures (Cao et al., 2014; Jia et al., 2009; Wang et al., 2012; Yu et al., 2012).

Although the negative consequences of losing the father can be mediated by direct effects on offspring, they might also be mediated indirectly, through adverse effects on mothers. However, the effects of fathers on their mates have received very little study. Exceptions include studies in Djungarian hamsters (*Phodopus campbelli*), which found that removal of the mate might influence pup survival by altering mothers' thermoregulatory abilities (Scribner and Wynne-Edwards, 1994; Walton and Wynne-Edwards, 1997; Wynne-Edwards and Lisk, 1989), as well as a recent study in prairie voles, which showed that mothers losing their mates have altered emotionality, including increased anxiety-related behavior in the elevated plus maze and more

passive stress-coping behavior in the forced-swim test (Bosch et al., 2017).

The California mouse is monogamous and biparental in both the field and lab, and fathers engage in all the same parental behaviors (except nursing), and to a similar extent, as mothers (Gubernick and Alberts, 1987). Most research in the California mouse has focused on the biology of paternal behavior; much less has focused on maternal care (Bester-Meredith et al., 2017), and the effects of fathers on their mates have received virtually no attention.

In this study we examined the effects of parenthood, as well as the effects of mate loss, on California mouse mothers. We hypothesized that mothers rearing offspring without assistance from their mates would have poorer morphological, physiological and affective condition, as well as impaired survival and development of pups, compared to mothers housed with their mates. In addition, we hypothesized that these effects of single motherhood would be further influenced by environmental conditions. We predicted that both single mothers and mothers housed with their mates would have lower body and fat mass, and display altered profiles of corticosterone (CORT), a metabolically important, stress-responsive hormone, compared to non-breeding females; and that the effects in single mothers should be even more severe than those in mothers housed with their male mate. We further predicted that paired mothers, but not single mothers, would show less anxiety-like and depression-like behavior than non-breeding females, as seen in other species. Finally, we anticipated that these differences between single and paired mothers would be more pronounced, or might only occur, under energetically challenging conditions that increase the cost of motherhood.

## **Material and Methods**

### **Animals**



California mice were bred in our colony at the University of California, Riverside (UCR) and were descended from mice from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Animals were housed in polycarbonate cages (44 × 24 × 20 cm) with aspen shavings as bedding and cotton as nesting material. Food (Purina 5001 Rodent Chow, LabDiet, Richmond, IN, USA) and tap water were provided *ad lib*. The colony was on a 14:10 light:dark cycle, with lights on at 05:00 h and lights off at 19:00 h. Room temperature was approximately 22° C, and humidity was about 57%. Cages were checked twice daily and changed weekly.

Sample sizes were based on prospective power analysis (G\*Power 3; Faul et al., 2009), using the magnitude of group differences and the within-group standard errors observed in a previous study of California mice (Saltzman et al., 2015). All procedures used were in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the UCR Institutional Animal Care and Use Committee. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

## **Experimental design**

Mice were weaned at 27-31 days of age, prior to the birth of younger siblings, and housed in same-sex groups of 3-4 related and/or unrelated, age-matched individuals. At 92-111 days of age ( $102.6 \pm 0.5$  days, mean  $\pm$  SE; young adulthood), 88 females were randomly assigned to two categories. Non-breeding females (NB, n=29) were housed with a male that had been vasectomized (see below) 7 days before pairing, and breeding females (n=59) were housed with an intact male. These latter males were vasectomized 16-21 days after pairing (13 to 32 days before birth of the first litter) to prevent postpartum pregnancies. Non-breeding

126 females were used to control for the effects of motherhood. Pair mates were no more closely  
127 related to each other than first cousins.

128 When its first litter was born, each breeding female was randomly assigned to one of two  
129 conditions: single mothers (SM, n=29), whose male mates were removed 1 day after the birth of  
130 the first litter, or paired mothers (PM, n=30), whose mates remained with them throughout the  
131 entire lactation period. All mothers (and NB, at a comparable time point) were then left  
132 undisturbed for 2-3 days until the beginning of the 25-day testing period (Fig. 1). At postpartum  
133 day 27 or 28, females and pups were weighed and their body composition was assessed (see  
134 below). Pups were also checked daily for eye opening (Harris et al., 2013; Zhao et al., 2018).

135 Approximately half of the pairs in each reproductive group (n=42: 14 NB, 14 PM, 14 SM)  
136 were housed under standard lab conditions throughout the experiment (CTRL). The remaining  
137 pairs (n=46: 15 NB, 16 PM, 15 SM) were housed from the time of pair formation in cages in  
138 which they were required to climb towers in order to gain access to food and water (CLIMB; see  
139 below; Fig. 2). Thus, we had a total of 6 experimental groups (3 reproductive groups  $\times$  2 housing  
140 conditions).

## 142 Climbing towers

143 A climbing-exercise paradigm modified from previous studies of house mice (Lionikas and  
144 Blizard, 2008; Mori et al., 2003), rats (Notomi et al., 2001) and California mice (Zhao et al.,  
145 2018) was used to subject the animals in the CLIMB condition to a mild physical challenge (Fig.  
146 2). Mice were housed in cages with plastic lids connected to two wire mesh (mesh openings:  $\sim$ 5  
147 mm  $\times$  5 mm) towers measuring  $\sim$ 8 cm in diameter and  $\sim$ 50 cm in height. Food was located at the  
148 top of one tower, and a water bottle was at the top of the other tower.

149

150 **Vasectomies**

151 Male mice were vasectomized using antiseptic techniques and standard surgical procedure

152 (Chauke et al., 2011). Briefly, mice were anesthetized with isoflurane gas using a vaporizer. The

153 incision region (1 cm above genital area) was shaved and sanitized, and a ventral midline

154 incision (approximately 1 cm) was made. The vas deferens was sutured and cut. All reproductive

155 structures were repositioned back in the abdominal cavity, the incision was closed with

156 absorbable sutures (Monocryl Suture 4-0 FS-2, Ethicon, San Angelo, TX) and the skin was

157 sealed using tissue glue (Vetbond Tissue Adhesive 1469SB, St. Paul, MN, USA).

158 Following surgery, males to be paired with non-breeding females were housed

159 individually in a standard cage for 7 days until pair formation. For breeding pairs, male and

160 female pair mates were housed for the first 7 days after surgery on opposite sides of a standard

161 cage divided in half by a steel mesh barrier, with food and water available in each half, to

162 prevent direct physical interaction during post-surgical recovery (Harris and Saltzman, 2013).

163 Males and females in non-breeding pairs were also housed in divided cages for 7 days at a

164 matched time point to control for any effects of separation from the pair mate. At the end of the

165 experiment, all females were euthanized and dissected to check for pregnancy. Two females

166 were found to be pregnant; therefore, their data were not used in any analyses.

167

168 **Measurements**

169 **Morphology**

170 **Body mass**

171 From the time of pair formation until the birth of the first litter in each breeding pair, or a

matched time point for NB, females were weighed to the nearest 0.01 g at 14:00-16:00 h twice weekly, at 3- to 4-day intervals, to assess overall health, habituate the animals to handling, and monitor pregnancies through patterns of mass gain. Females were also weighed at 14:00-16:00 h on test days 1, 4, 8, and 13, as well as at 11:40-12:30 h on test day 12 (Fig. 1).

### **Body composition (test days 1, 4, 8, 13)**

Body composition was assessed with an EchoMRI-100 magnetic resonance whole-body analyzer (Echo Medical Systems, Houston, TX, USA) as previously described (Zhao et al., 2017, 2018). Each scan lasted approximately 90 s, and animals were not sedated or anesthetized. Lean and fat masses were computed both as absolute values and as percentages of total body mass (analyses using body mass as a covariate yielded similar results).

### **Euthanasia and organ collection (test day 14)**

Females were decapitated between 13:30 and 16:00 h. Trunk blood was collected in a heparinized weighing boat, and organs [heart, liver, spleen, leg muscles (left and right triceps surae), ovaries (left and right), uteri, adrenal glands (left and right) and kidneys (left and right)] were removed and weighed as previously described (Zhao et al., 2017, 2018). At a later date, stomach, caecum, and large and small intestines were thawed, cut open, cleaned of internal contents, blotted, and weighed as previously described (Andrew et al., 2016).

### **Behavioral indicators of neophobia, anxiety, and depression**

#### **Novel-object test (test day 4)**

As described above, mothers in some mammalian species, such as rats and mice, exhibit reduced

anxiety and fearfulness, as well as blunted responses to stressors, during late pregnancy and lactation (Brunton et al., 2008; Lightman et al., 2001; Slattery and Neumann, 2008). We evaluated whether single motherhood would further influence mothers' anxiety/fearfulness using the novel-object test, which is commonly employed to assess neophobia in small mammals (Chauke et al., 2012; Greggor et al., 2015; Sarowar et al., 2016). In this test, frequency and duration of an animal's interactions with an unfamiliar object are considered negative indices of neophobia and, correspondingly, of fear and/or anxiety (Hughes, 2007; Powell et al., 2004). Novel-object tests were administered between 14:00 h and 16:00 h. Each female's cage mate(s) (male mate and/or pups) were removed from the home cage, housed in a clean cage, and moved to a separate room. After 5 min, a golf ball (diameter: 4.8 cm) was placed in the corner of the home cage farthest from the female. Behavioral responses to the novel object were video-recorded for 5 min. Immediately after testing, the female was weighed, scanned for body composition (see above), and then reunited with its cage mate(s) in the home cage. Behavioral parameters scored included latency to approach to within 2 cm of the golf ball, bouts of approaching the ball, total duration of immobility, and total durations of time spent sniffing and touching the ball (Chauke et al., 2012).

#### **Open-field test (test day 8)**

The open-field test is a standard method for measuring anxiety-like behavior in rodents, based on findings that many pharmacological anxiolytic treatments reduce measures of anxiety-like behaviors in this paradigm (reviewed in Prut and Belzung, 2003). The amount of exploratory behavior and time spent in the center of the arena are considered to be negative measures of anxiety, while the amount of defecation is considered a positive measure (Archer, 1973; Flint et

al., 1995; Willis-Owen et al., 2007). We administered tests between 14:00 h and 16:00 h. The open-field arena was a 1.0 m × 1.0 m square with a height of 0.4 m, constructed of opaque black plastic and placed on a clean sheet of white butcher paper to enhance contrast between the arena floor and the darkly colored mice as previously described (Perea-Rodriguez et al., 2018). Tests were recorded by a GoPro HERO SESSION video camera (GoPro Inc., San Mateo, CA, USA) suspended above the arena. After each test, the arena was disinfected and the butcher paper was replaced. The open-field arena was located in an environmental chamber maintained at 1400 lux with two overhead white lights; temperature and humidity were maintained at ~23°C and ~70%, respectively. For each test, the female subject was placed in the center of the arena and video-recorded for 10 min. Immediately following testing, the female was weighed, scanned for body composition (see above), and then reunited with its cage mate(s) in the home cage.

Exploratory behaviors in the open-field arena were quantified using TopScanLite software (Clever Sys Inc., Reston, Virginia, USA). The arena was divided into two concentric regions in the software: an inner square, measuring 0.5 × 0.5 m, in the center of the arena, and an outer region extending 0.5 m from each wall to the perimeter of the inner square. Bouts of crossing the boundary between inner and outer regions, total distance moved, distance moved in each region, and duration of time spent in each region were determined. We also recorded the number of fecal boli produced during the 10 min of testing. Distance traveled, duration in the inner region of the arena, and number of crossing bouts were considered negative indicators of anxiety-like behavior, whereas number of fecal boli was considered a positive indicator (Gould et al., 2009).

#### **Tail-suspension test (test day 12)**

The tail-suspension test is widely used to evaluate depression-like behavior in rodents, with the duration of immobility considered a positive index of depression; numerous antidepressant medications reduce immobility in this paradigm (reviewed in Cryan et al., 2005, Yan et al., 2010). We used the tail-suspension test both to assess depression-like behavior and as an acute stressor for assessment of the CORT stress response.

Between 11:30 and 12:35 h, mice were suspended by their tails from a ring stand for 6 min and the duration of immobility was measured, as previously described (Zhao et al., 2017, 2018). Briefly, the ring stand was placed on an activity detector unit (MAD-1: Sable Systems International, Henderson, NV, USA) interfaced to a Macintosh computer equipped with an A-D converter and Warthog LabHelper software (<http://www.warthog.ucr.edu>). Activity was recorded every 0.004 s. Warthog LabAnalyst software (<http://www.warthog.ucr.edu>) was used for baseline correction and calculation of activity duration (Malisch et al., 2009). Duration of time spent immobile was interpreted as a positive indicator of depression-like behavior (Cryan et al., 2005). In addition, we recorded the number of fecal boli produced during the 6 min of testing as a positive indicator of anxiety.

#### **Saccharin preference test (test day 6)**

Preference for sweet solutions is frequently used as a measure of anhedonia, a common marker of depression. The relative amount of sweet solution consumed is considered a negative index of anhedonia (Moreau, 1997; Willner et al., 1992). This test has consistently demonstrated a decrease in consumption of, and preference for, highly palatable sweet solutions under chronically stressful conditions (Grippe et al., 2005; Papp et al., 1991; Strekalova et al., 2004). We characterized females' preference for saccharin (Sweet'N Low; Cumberland Packing Corp.,

264 Brooklyn, NY, USA) solution (0.2% w/v in water) vs. water as previously described (Zhao et al.,  
265 2017, 2018). Briefly, each mouse's cage mate(s) were removed from the home cage at the time  
266 of lights-off (19:00 h). The female remained alone in its home cage for 4 h, with access to  
267 standard chow and two plastic syringes, one containing ~35 ml of water and the other containing  
268 ~35 ml of 0.2% saccharin solution. Positions of the two types of liquid were randomly assigned.  
269 The syringes were weighed immediately before and after the 4-h test period. The amount of  
270 consumed saccharin solution divided by the overall liquid consumption was calculated as an  
271 index of relative preference for saccharin solution.

272

### 273 **Home-cage behavioral observation (test days 5, 7, 9, 11)**

274 Females were video-recorded in their home cages under undisturbed conditions for 1 hour on test  
275 days 5 (20:00-22:00 h, under red light), 7 (14:00-16:00 h), 9 (08:00-10:00 h), and 11 (02:00-  
276 04:00 h, under red light). We quantified total duration of time spent in each of six mutually  
277 exclusive behavior categories - feeding, drinking, nest building, sleeping (sedentary with eyes  
278 closed or with face tucked into body and without pups underneath), resting (sedentary, not  
279 associated with other behaviors), and maternal behaviors, including nursing (sedentary with  
280 visible pups underneath), grooming pup, retrieving pup – using The Observer software (Noldus,  
281 Wageningen, Netherlands).

282

### 283 **Blood collection (test days 2, 3, 10, 12)**

284 Blood was collected under baseline conditions on test days 2 (03:50-04:10 h), 3 (19:50-20:10 h)  
285 and 10 (11:50-12:10 h) for analysis of baseline CORT concentrations across the diel cycle, and  
286 immediately after tail-suspension tests (11:30-12:35 h) on test day 12 for assessment of the



CORT response to an acute stressor. Animals were anesthetized with isoflurane, and blood was collected from the retro-orbital sinus into heparinized capillary tubes within 3 min after either initial disturbance to the cage (baseline samples) or the end of the tail-suspension test (post-stress samples). Blood was centrifuged immediately for 12 min (13,300 rpm, 4 °C), and plasma was removed and stored at -80 °C.

### **Corticosterone radioimmunoassay**

Plasma corticosterone concentrations were determined using a radioimmunoassay kit (cat. no. 07120103; MP Biomedicals, Solon, OH) previously validated for *P. californicus* (Chauke et al., 2011). The assay was run according to manufacturer's instructions, but the assay standard curve was extended down from 25 to 12.5 ng/ml (90-91% bound) and went to 1000 ng/ml (20-21% bound). Samples were diluted anywhere from 1:100 to 1:1600 in order to ensure that values were contained within the curve. All samples were run in duplicate. Intra- and inter-assay coefficients of variation, calculated using kit-provided controls, were 2.19% and 5.31% for the high control, and 9.48% and 7.82 for the low control, respectively.

### **Statistical analysis**

Data were analyzed by analysis of covariance (ANCOVA) or repeated-measures analysis of variance (ANOVA) and Fisher's LSD for post hoc tests using SPSS. Age and/or other potentially relevant variables (see Results) were used as covariates. For each analysis, residuals were checked for skewness and visually inspected for outliers. Plasma CORT data were also tested for sphericity using Mauchly's test, and all other traits were tested for homogeneity of variance using Levene's test. Dependent variables were transformed as needed; however, data are presented as

non-transformed values in the text and figures for ease of interpretation. For paired (right and left) organs, values from the two organs were compared using a paired t-test and a Pearson correlation to gauge repeatability (Table 1), and mean values were used for subsequent analyses. For the breeding pairs, we also performed 2-way (reproductive group x housing condition) ANOVAs to determine if litter size, pups' age of eye opening, and body composition at weaning age differed between pups reared by single and paired mothers or between pups in the CTRL and CLIMB housing conditions.

Excluding such nuisance variables as age, this study generated 400 P-values, 96 of which were <0.05. These tests include a substantial amount of non-independence because the same individuals were measured for all traits, some traits were correlated, and many tests were inter-related. To compensate for non-independence in multiple related tests, we used the Adaptive False Discovery Rate procedure as implemented in PROC MULTTEST in SAS 9.4 (SAS Incorporated, Cary, NC, USA). Based on this procedure, the 71 smallest P-values would have adjusted P-values <0.05 (the highest being 0.016). For simplicity, all P-values reported in the text and figures are raw values, not adjusted for multiple comparisons. We calculated partial eta squared ( $\eta^2$ ) effect sizes for all ANOVA and ANCOVA, and Cohen's d effect sizes for all pairwise comparisons with  $P < 0.05$ . We refer to P-values  $\leq 0.016$  and those between 0.016 and 0.05 with partial  $\eta^2 > 0.06$  or Cohen's  $d > 0.5$  as 'significant'. Effect size of  $0.06 < \eta^2 < 0.14$  and  $0.5 < d < 0.8$  is considered a medium effect, and  $\eta^2 > 0.14$   $d > 0.8$  is considered a large effect (Cohen, 1988). All tests were two-tailed.

## Results

### Home-cage behavior

Each category of behavior (feeding, drinking, nest building, resting, sleeping, and maternal behaviors) was analyzed separately for each of the four time points / test days. Duration of feeding differed among reproductive groups ( $P = 0.029$ ) only at 02:00-04:00 h (near the end of the active [dark] period; day 11), with SM spending more time feeding than NB ( $P = 0.008$ ,  $d = 0.70$ ). Feeding behavior did not differ among housing conditions and was not affected by a housing condition  $\times$  reproductive group interaction at any time point. Duration of drinking, as well as nest building, did not differ reliably among reproductive groups, between housing conditions, or by a housing condition  $\times$  reproductive group interaction at any time point.

Time spent resting differed among reproductive groups at all four time points (all  $P < 0.005$ ), with NB spending more time resting than both PM (all  $P < 0.020$ , all  $2.50 > d > 0.5$ ) and SM (all  $P < 0.003$ , all  $2.00 > d > 0.5$ ). Time spent sleeping was also higher in NB than both PM ( $P = 0.014$ ,  $d = 0.52$ ) and SM ( $P = 0.005$ ,  $d = 0.65$ ) at 14:00-16:00 h.

No significant effects of reproductive group (SM vs. PM), housing condition or housing condition  $\times$  reproductive group interaction were found on total duration of time spent in maternal behavior (nursing, grooming pup, retrieving pup) at any time point.

### **Behavioral indicators of neophobia, anxiety, and depression**

#### **Novel-object test**

Neither housing condition, reproductive group, nor their interaction affected females' latency to approach to within 2 cm of the novel object (golf ball), number of approaches, duration of time spent sniffing and touching the novel object, or duration of immobility (Supplementary Table 1).

#### **Open-field test**

ANCOVA (with age as a covariate) revealed a significant difference among reproductive groups in number of fecal boli produced ( $P = 0.014$ ), with both SM and PM producing more boli than NB ( $P = 0.009$ ,  $d = 0.69$ ;  $P = 0.012$ ,  $d = 0.62$ , respectively; Fig. 3). Number of fecal boli was not influenced by a main effect of housing condition or a housing condition  $\times$  reproductive group interaction.

Neither housing condition, reproductive group, nor their interaction affected females' distances traveled during the open-field test (total distance and distance within each region), duration of time spent in the inner or outer region, or number of crossing bouts (Supplementary Table 1).

#### **Tail-suspension test**

ANCOVA (with age and body mass as covariates) revealed a positive effect of body mass ( $P = 0.012$ ) on the duration of immobility, but we found no significant differences between housing conditions or among reproductive groups, and the housing condition  $\times$  reproductive group interaction was not significant.

As in the open-field test, number of fecal boli expelled differed significantly among reproductive groups, with both SM and PM producing more boli than NB ( $P = 0.036$ ,  $d = 0.76$ ;  $P = 0.030$ ,  $d = 0.73$ , respectively). Number of fecal boli was not influenced by a main effect of housing condition or a housing condition  $\times$  reproductive group interaction (Table 2; Fig. 3).

#### **Saccharin-preference test**

ANCOVA (with age as a covariate) showed that preference for saccharin solution did not differ among reproductive groups or between housing conditions, and was not affected by a housing

condition × reproductive group interaction (Supplementary Table 1).

## **Plasma corticosterone concentrations**

### **Baseline corticosterone**

Baseline CORT concentrations were analyzed via repeated-measures ANOVA with time of sample (04:00, 20:00, and 12:00 h) as a within-subjects factor, and housing condition and reproductive group as between-subjects factors. The data were not normally distributed, mainly because of 8 outliers (2 CTRL SM, 2 CTRL PM, 2 CTRL NB, and 2 CLIMB SM) for samples collected at 04:00 h (outlier values were ~10-fold higher than other samples, for no obvious biological reason). Therefore, data from the outliers were discarded, and the remaining data were log<sub>10</sub>-transformed to meet normality assumptions; however, analysis of log<sub>10</sub>-transformed data yielded similar results whether the outliers were included or not. Here we report results of analyses with the outliers omitted.

Plasma CORT concentrations (Fig. 4) varied across the three times of day (main effect of time:  $F_{2,112} = 242.11$ ,  $P < 0.001$ ) in a pattern that was influenced by both housing condition (time × housing condition interaction:  $F_{2,112} = 3.76$ ,  $P = 0.026$ ) and reproductive group (time × reproductive condition interaction:  $F_{2,112} = 3.45$ ,  $P = 0.011$ ). CORT levels were highest around the beginning of the active period (20:00 h, 1 h after lights-off) and lowest near the beginning of the inactive period (04:00 h, 1 h before lights-on). Neither the main effects of housing condition or reproductive group, the interaction between housing condition and reproductive group, nor the 3-way interaction between time, housing condition and reproductive group was significant.

Post-hoc tests for the interaction between time and housing condition revealed that at 04:00 h, females in standard cages (CTRL) had significantly higher CORT levels than females in

towered cages (CLIMB;  $P = 0.009$ ,  $d = 0.72$ ). Post-hoc tests for the interaction between time and reproductive condition revealed that at 20:00 h, both single mothers and paired mothers had lower CORT levels than non-breeding females ( $P = 0.002$ ,  $d = 0.95$ ;  $P = 0.040$ ,  $d = 0.52$ , respectively). In addition, at 12:00 h, single mothers had higher baseline CORT levels than non-breeding females ( $P = 0.030$ ,  $d = 0.82$ ). No other pairwise comparisons were significant.

### **Corticosterone response to stress**

Data were analyzed using repeated-measures ANOVA with stress condition (baseline CORT at 12:00 h, post-stress CORT [immediately after a 6-min tail-suspension test] at 12:00 h) as a within-subjects factor, and housing condition and reproductive group as between-subjects factors. The tail-suspension test significantly increased  $\log_{10}$ -transformed plasma CORT levels (main effect of stress:  $F_{1,66} = 345.43$ ,  $P < 0.001$ ), and this effect differed among reproductive groups (stress  $\times$  reproductive group interaction:  $F_{66} = 4.48$ ,  $P = 0.015$ ; Fig. 5). As described above, post-hoc tests indicated that baseline CORT levels at 12:00 h were significantly higher in single mothers than in non-breeding females. However, post-stress CORT levels were significantly lower in single mothers ( $P = 0.011$ ,  $d = 0.73$ ), but not paired mothers ( $P = 0.130$ ), than in non-breeding females. None of the remaining main effects (housing condition, reproductive group) or interactions (stress  $\times$  housing condition, stress  $\times$  housing condition  $\times$  reproductive group) were significant.

### **Morphology**

#### **Body mass**

We analyzed each individual body-mass value during the test period (days 1, 4, 8, 12 and 13) and

also the mean postpartum body mass from all test days except day 12. Body mass on day 12 was excluded because it was measured immediately after the tail-suspension test; however, analyses that included test day 12 generated similar results. ANCOVA (with age as a covariate) found no main effect of housing condition, reproductive group, or interaction between housing condition and reproductive group for either mean body mass or body mass on test days 1, 4, 8 or 13. On test day 12, body mass differed among reproductive groups ( $P = 0.046$ ); post-hoc tests revealed that SM had significantly higher body mass than NB ( $P = 0.014$ ,  $d = 0.75$ ; Table 2).

### **Body composition**

Neither rank-transformed fat mass on any test day nor mean fat mass differed between housing conditions or among reproductive groups, when using age as a covariate. Similar results were found when mean or individual fat mass was expressed as percentage of total body mass, except that rank-transformed percent fat mass on test days 8 and 13 showed a main effect of reproductive group ( $P = 0.014$  and  $P = 0.031$  respectively), with SM having lower percent fat mass than both PM ( $P = 0.043$ ,  $d = 0.64$ ) and NB ( $P = 0.005$ ,  $d = 0.74$ ) on test day 8, and SM having lower percent fat mass than NB ( $P = 0.011$ ,  $d = 0.66$ ) on test day 13. Note that mean and individual fat mass, as well as percent fat mass, had significant heterogeneity of variance that was not eliminated by transforms (with NB having higher variability than SM and PM), but the differences among groups are evident.

Square root-transformed mean postpartum lean mass differed among reproductive groups ( $P = 0.029$ ; Fig. 6) when analyzed by ANCOVA with age as a covariate, with SM having higher lean mass than NB ( $P = 0.008$ ,  $d = 0.74$ ). Lean mass also differed among reproductive groups for test days 4 and 8 ( $P = 0.003$  and  $P = 0.001$ , respectively). On each of these days, both SM and

PM had significantly higher lean mass than NB ( $P = 0.001$ ,  $d = 1.02$ ;  $P = 0.016$ ,  $d = 0.70$ , respectively). No differences were found among groups for test day 1 or 13.

When expressed as a percentage of total body mass, mean percent lean mass did not differ among reproductive groups. However, percent lean mass on test days 4 and 13 differed among reproductive groups ( $P = 0.008$  and  $P = 0.050$ , respectively), with SM having higher percent lean mass than NB ( $P = 0.002$ ,  $d = 0.84$ ) on test day 4, and both SM and PM having higher percent lean mass than NB ( $P = 0.042$ ,  $d = 0.58$ ;  $P = 0.022$ ,  $d = 0.58$ , respectively) on test day 13. Percent lean mass did not differ among groups on any other day. Neither lean mass nor percent lean mass was influenced by a main effect of housing condition or by a housing condition  $\times$  reproductive group interaction (Supplementary Table 1).

## **Organ masses**

Body mass was used as a covariate in all organ-mass analyses. For all paired organs (triceps surae, ovaries, adrenals and kidneys), the two organs showed high correlations within individual animals, similar to findings in male California mice (Andrew et al., 2017; de Jong et al., 2013; Zhao et al. 2017, 2018). Right kidneys were significantly heavier than left kidneys (Table 1), as has been seen in other mammals and with other organs (e.g. Coleman et al., 1998; Idelman, 1978), while right adrenals were significantly lighter than left adrenals. Three females from the CLIMB condition had much higher triceps surae mass than the other CLIMB females and were excluded from analysis as outliers.

ANCOVA (with age and body mass as covariates) found significant positive effects of body mass (all  $P < 0.001$ ) and significant effects of reproductive group on mass of the heart ( $P = 0.023$ ), liver ( $P < 0.001$ ), stomach ( $P = 0.003$ ), caecum ( $P = 0.005$ ), large intestine ( $P = 0.006$ ),



small intestine ( $P = 0.014$ ), and uterus ( $P = 0.004$ ), and on mean mass of the triceps surae ( $P = 0.008$ ) and ovaries ( $P = 0.002$ ) (Table 2; Fig. 6; Fig. 7). No main effects of housing condition or housing condition  $\times$  reproductive group interactions were found.

Post-hoc tests revealed that SM had significantly higher heart mass ( $P = 0.006$ ,  $d = 1.14$ ) and caecum mass ( $P = 0.001$ ,  $d = 1.33$ ), and lower rank-transformed triceps surae mass, than NB ( $P = 0.002$ ,  $d = 0.25$ ).  $\text{Log}_{10}$ -transformed liver mass was higher in SM than in both PM ( $P = 0.046$ ,  $d = 0.61$ ) and NB ( $P < 0.001$ ,  $d = 1.32$ ) and was higher in PM than NB ( $P = 0.023$ ,  $d = 0.66$ ). NB had lower stomach mass, large intestine mass, and small intestine mass compared to both SM ( $P = 0.001$ ,  $d = 1.33$ ;  $P = 0.037$ ,  $d = 0.76$ ;  $P = 0.014$ ,  $d = 1.07$ , respectively) and PM ( $P = 0.014$ ,  $d = 0.82$ ;  $P = 0.002$ ,  $d = 0.91$ ;  $P = 0.009$ ,  $d = 0.96$ , respectively). Finally, SM had lower uterus mass than PM ( $P = 0.001$ ,  $d = 0.90$ ) and lower ovary mass than both PM ( $P = 0.003$ ,  $d = 0.66$ ) and NB ( $P = 0.001$ ,  $d = 0.70$ ). No other organ masses or lengths differed among reproductive groups or between housing conditions, and no significant or significant interactions were found (Supplementary Table 1).

## **Pup development**

Neither litter size at weaning (range: 1–3 pups, mean  $\pm$  SE =  $1.9 \pm 0.1$ ) nor day of eye opening of first pup in each litter ( $16.4 \pm 0.4$  days) differed between offspring of single mothers and paired mothers or between the CTRL and CLIMB conditions. We also found no difference in pups' body mass or body composition at the time of weaning (postpartum day 27 or 28) (Table 3).

## **Discussion**

In this study, we tested the hypothesis that mate loss would lead to poorer morphological,

physiological and affective condition in California mouse mothers, as well as impaired survival and development of pups, compared to mothers housed with their mates. We tested this hypothesis under both standard laboratory conditions and mildly challenging conditions, in which mice had to climb wire-mesh towers to obtain food and water. Because females housed in towered cages had to spend more time and energy to climb and forage for food, we expected to find more severe effects of being single under the challenging condition. We found that mothers that lost their mates shortly after giving birth showed more morphological, physiological and behavioral differences from non-breeding females than did females whose male mates were present throughout the lactational period; however, these effects were not modulated by housing condition.

#### **Effects of housing condition**

Housing under challenging conditions had a moderate effect on baseline CORT concentrations. As previously found in California mice (Harris et al., 2012) and many other species (Dallman et al., 1987; Dickmeis, 2009), CORT levels were highest around the start of the active period (20:00 h), intermediate midway through the active period (12:00 h), and lowest at the end of the active period (04:00 h). Although this pattern was seen in virtually all females in the three reproductive groups, mice in standard cages had significantly *higher* plasma CORT levels than those in towered cages at 04:00 h. A previous study in our lab showed that chronic variable stress could increase baseline CORT levels in male California mice at 09:00 h (Harris et al., 2013). In contrast, housing in towered cages in the present study did not increase CORT levels. Instead, the mice housed in standard cages had a higher CORT trough compared to the ones in towered cages, causing a more flattened pattern of the CORT rhythm. An increase in glucocorticoid

levels at the circadian trough is commonly seen under chronic stress, which, in humans, might facilitate development of the metabolic syndrome (Dallman et al., 2000) and depression (Meijer et al., 1997) in the long term. However, it is unclear what the more dynamic rhythm means in mice housed in towered cages.

However, we found no difference in the preference for saccharine between females housed in towered cages and those housed under standard conditions. Low preference for sweetened water or other rewarding substances may be an indicator of anhedonia, a component of depression-like behavior (Schrader, 1997). Stressful environmental conditions, such as restricted access to food, continuous overnight illumination, cage tilt, and intermittent white noise, can decrease responsiveness to rewards in lab rats (Willner et al., 1992) and mice (Strekalova et al., 2004). Environmental enrichment, similarly, can influence animals' preference for rewarding stimuli. For example, rats housed in enriched environments show reduced cocaine self-administration (Puhl et al., 2012). It is possible that the required amount of climbing in the towers in our study might not have been energetically demanding, in view of the California mouse's semi-arboreal lifestyle in the wild (Ribble, 1992). However, whether the towered cages in our study acted as a stressor or as enrichment is not clear. In any case, we found no other effects of towered cages, aside from changes in baseline CORT, suggesting that this housing paradigm is neither highly stressful nor particularly enriching for California mice. In a recent study of male California mice, we found other effects of being housed in these towered cages (Zhao et al., 2018), including increases in body mass and body fat, but those animals were also food-restricted every third day, which likely accounted for the effects of housing conditions.

## **Effects of motherhood**

Compared to non-breeding females, both single mothers and paired mothers produced significantly more fecal boli in the open-field and tail-suspension tests, which is considered a positive indicator of anxiety level (Archer, 1973; Bronikowski et al., 2001; Colman et al., 2007; Flint et al., 1995). Motherhood in several uniparental mammals influences females' responses to stress: mothers exhibit blunted hormonal, neural and behavioral responses to a multitude of stressors during pregnancy and lactation (Brunton et al., 2008; Lightman et al., 2001; Slattery and Neumann, 2008), including reduced anxiety and fearfulness (Lonstein, 2007; Slattery and Neumann, 2008), potentially to protect maternal care from being inhibited by stress (Brunton et al., 2008). In contrast, we found evidence that mothers were more anxious than non-breeding females. This might indicate a difference among rodent species or perhaps between uniparental and biparental animals, but more studies on other biparental species are needed to test this hypothesis. Alternatively, since we found no other effects of motherhood on behavior in the novel-object, open-field or tail-suspension tests, the difference in production of fecal boli might reflect changes in bowel function associated with lactation (Elias and Dowling, 1976; Hammond, 1997). Indeed, mothers in our study had larger intestines, caeca, and stomachs, which could be associated with the differences in production of fecal boli.

Mothers in our study, both paired and single, had higher lean mass on test days 4, and 8 than non-breeding females. We also found lower percent fat and higher percent lean masses in single mothers and, in some cases, in paired mothers than in non-breeding females on one or more test days. Although no measures of body composition differed significantly among reproductive groups on test day 1 (3-4 days after the mothers gave birth), the differences were mostly significant beginning on test day 4, indicating that lactation increased mothers' lean mass while decreasing fat stores, but that this effect did not become apparent within the first day after

parturition.

Energy balance in female mammals is strongly influenced by reproduction; females have high energy demands during pregnancy and, especially, lactation (Boland et al., 2001; Speakman, 2008). Motherhood can induce changes in body composition by increasing utilization of fat stores, especially in capital breeders, which rely primarily on fat stores to meet the energetic demands of reproduction (Bonnet et al., 1998). Our observed effects of motherhood on body fat and lean content in California mice are similar to those in capital breeders. Whether California mice are capital or income breeders (increase food intake to meet higher energetic demands) has yet to be investigated, but one study suggested that females in the congeneric species *P. leucopus* (white-footed mouse) are likely to be income breeders (Millar, 1975).

#### **Effects of single motherhood**

In small mammals, mothers have high basal and/or resting metabolic rate and experience growth of the alimentary tract in order to meet the high energy demands of lactation (Speakman, 2008). Reproduction has been found to induce a higher mass in liver, stomach, and large and small intestine compared to nonreproducing groups (Sadowska et al., 2018). Although we did not measure metabolic rate in this study, we found that single mothers had significantly greater heart mass than non-breeding females. Heart mass is usually positively correlated with basal metabolic rate (Chappell et al., 1999; Daan et al., 1990; Meerlo et al., 1997), daily energy expenditure (Dlugosz et al., 2012), and maximal oxygen consumption (Andrew, 2017) in vertebrates. Measures of mothers' metabolic rates in future studies would be helpful to further clarify the physiological effects of single motherhood.

Liver is a key organ that governs glucose and lipid metabolism as well as numerous other

functions (Samuel and Shulman, 2016). In our study, mothers had heavier livers than non-breeding females, consistent with findings from other species that lactation can induce hepatic growth (Speakman, 2008). In addition, we found that liver mass was higher in single mothers than in paired mothers, which might indicate that single mothers have higher energy demands during lactation than paired mothers (Piersma, 1997). However, we did not find significant differences in mass of any part of the gastrointestinal tract (stomach, caecum, large intestine, or small intestine) between single and paired mothers, although both groups had significantly higher values than non-breeding females. Thus, elevated liver mass in mothers, especially single mothers, might be associated with differences in functions separate from the gastrointestinal tract, such as lipid and carbohydrate metabolism, sulfation or bile formation (Gebhardt, 1992). Alternatively, although both heart mass and liver mass can correlate positively with metabolic rate and energetic demands, thickened heart muscle and liver enlargement can also have pathological causes and/or consequences, such as hypertension and fatty liver disease, in both humans and lab rodents (Samuel and Shulman, 2017; van Nierop et al., 2013). In addition, heart and liver can influence each other's functioning, and many metabolic factors can affect both organs simultaneously (Møller and Bernardi, 2013). Thus, the proximate causes and consequences of heart and liver enlargement in single mothers are not clear.

Triceps surae is an important muscle in running behavior, and the mass of gastrocnemius and/or triceps surae typically is positively related to sprinting performance in mice (Dohm et al., 1994; Syme et al., 2005). Research in exercise physiology indicates that voluntary exercise (e.g., wheel-running) in mice leads to increases in skeletal muscle masses (Soffe et al., 2016). Single mothers in our study had significantly lower triceps surae mass than non-breeding females, possibly due to changes in their behavior, such as less locomotion and more huddling with their

pups.

We did not observe differences in triceps surae mass between females in standard and towered cages. In house mice, towers similar to the ones used in the present study have revealed a strain  $\times$  exercise interaction in the forelimb biceps brachii muscle, but no effect of climbing exercise was found on hindlimb muscles (Lionikas and Blizard, 2008; Mori et al., 2003). In contrast, a similar study found that hindlimb muscles of rats housed in towered cages were heavier than those of control rats after 4 weeks of exercise, but not after 8 weeks of exercise (Notomi et al., 2001). The different findings from rats, house mice, and California mice might be accounted for by possible species differences in behavioral responses to the towers and/or by differences in the duration of housing in towered cages.

Single mothers in our study had significantly lower ovarian and uterine masses compared to paired mothers, possibly due to lack of stimulation from a (vasectomized) male mate. Masses of reproductive organs in females are commonly used as indicators of reproductive function (Kumar et al., 2000; Kumar, 2005; Lee et al., 1998) and can be influenced by several reproductive hormones (e.g. luteinizing hormone, follicle-stimulating hormone, estrogen; Dewailly et al., 2006; Dupont et al., 2000; Halpin and Charlton, 1988; Kumar et al., 1997). Secretion of these hormones, in turn, can be elicited by interactions with males, including mating behaviors (Edwards, 1970). In some rodent species, for example, mating with a sterile male can induce pseudopregnancy. Although pseudopregnancy has not been studied in California mice, it is possible that paired mothers and non-breeding females, but not single mothers, might have been pseudopregnant during the period of data collection. Additionally or alternatively, reduced ovarian and uterine masses in single mothers might have resulted more directly from increased energetic demands on these females and shunting of metabolic resources to other, more essential

organs such as heart and liver. Indeed, energetic demands can greatly influence reproductive function in female mammals at the level of the ovaries and uterus through changes in lipid metabolism (Bellefontaine and Elias, 2014; Fontana and Torre, 2016).

Single and paired mothers showed differences in duration of feeding behaviors in the home cage. Single mothers, but not paired mothers, spent more time feeding than non-breeding females at 02:00-04:00 h, indicating that single mothers may need higher food intake to meet the higher energetic demands of caring for offspring without assistance from a mate.

We found no effects of single motherhood on behavior in the novel-object, open-field or tail-suspension tests, compared to paired mothers. In contrast, a recent study in prairie voles found that mothers whose male mates were removed on post-pairing day 18, prior to the birth of their first litter, showed altered emotionality, including increased anxiety-related behavior in the elevated plus maze and more passive stress-coping in the forced-swim test, which might result from increased signaling in the brain's corticotropin-releasing factor system (Bosch et al., 2017). This might reflect a difference between California mice and prairie voles. The timeline regarding the male-female separation might also explain the different findings in the two studies – while single mothers in the present study were separated from their male mate on the first day after parturition, approximately 35 days after pair formation, the prairie vole mothers were separated from their mate prior to giving birth. The earlier and longer separation from their mate might have been more stressful for the prairie vole mothers, which might have resulted in more severe emotional effects of single motherhood.

### **Corticosterone**

Along with many other hormonal changes during lactation, postpartum females in



uniparental rodents and humans can have elevated baseline glucocorticoid levels in order to maintain milk production and increase food intake, facilitating shifts in feeding behavior and energy utilization to meet increased metabolic demands (Tu et al., 2005; Woodside et al., 2012). In the present study, we found that single mothers had higher baseline CORT levels at 12:00 h than non-breeding females. However, at 20:00 h (around the peak of the CORT rhythm), both single and paired mothers had significantly lower CORT levels than non-breeding females, which generated a more flattened CORT rhythm in mothers. Interestingly, lactating rats also have a more flattened CORT rhythm than virgin females, although the flattening in that species occurs through an elevation of the nadir CORT level (Walker et al., 1992).

In addition to baseline CORT concentrations, CORT responses to an acute stressor differed among reproductive groups. A 6-minute tail-suspension test midway through the active period markedly elevated plasma CORT levels in all animals, as expected, but this effect was blunted in single mothers, compared to non-breeding females: single mothers had significantly higher CORT levels under baseline conditions, but significantly lower CORT levels immediately after the stressor. Neither group differed significantly from paired mothers either in baseline CORT levels at 12:00 or in stress-induced CORT levels, consistent with a previous study in our lab (Chauke et al., 2011). The effect of single motherhood on CORT responses to an acute stressor, compared to non-breeding females, contrasts markedly with the lack of effects on behavioral indices of emotionality, as described above.

Lactating females in several species (e.g., rats, sheep) are less likely than non-lactating females to exhibit glucocorticoid elevations in response to stressful stimuli (Tu et al., 2005). On the other hand, a study of a biparental primate, the common marmoset (*Callithrix jacchus*), found no differences in cortisol responses to acute stressors between paired, reproductively

experienced females that were and were not lactating (Saltzman and Abbott, 2011). Our findings of blunted CORT responses in single but not paired mothers might indicate that, with help from the male mate, motherhood in biparental species differs in fundamental ways from motherhood in uniparental species, therefore not requiring marked changes in functioning of the hypothalamic-pituitary-adrenal (HPA) axis. Removal of the mate might increase the demands on mothers in biparental species, thereby mimicking the situation in uniparental species and eliciting changes in HPA activity, similar to findings in several bird species (Wingfield and Sapolsky, 2003). For example, removal of the mate might alter mothers' food intake, activity levels, sleep patterns, thermoregulatory abilities (Walton and Wynne-Edwards, 1997), or patterning of nursing bouts, all of which could potentially affect baseline CORT levels and responses to stress.

#### **Pups**

None of the measures that we monitored in pups – survival, age of eye opening, and body mass and body composition at the age of weaning – differed between pups reared by single mothers and those reared by both mother and father. This result indicates that single mothers were able to provide a similar quality of parental care and raise a similar quality and number of offspring, compared to paired mothers, both under standard housing conditions and when housed in towered cages. However, it is possible that effects of having a single mother on the offspring could be revealed under more challenging conditions. Losing fathers in California mice has been found to impair pup survival and development in the field and in energetically challenging laboratory settings, but not under standard lab conditions (Bredy et al., 2007; Cantoni Brown, 1997; Dudley, 1974; Gubernick and Teferi, 2000; Gubernick et al., 1993; Wright and Brown,

2002). Therefore, it appears that mothers in this species can compensate for the loss of a mate by providing more maternal care, under benign living conditions but not under demanding conditions, in order to maintain offspring survival and quality. In contrast, in prairie voles, single mothers showed no evidence of strong parental compensation in response to the absence of the father, indicating a minimal effect of family structure on maternal behavior but a large effect on the total amount of pup care (Ahern et al., 2011; Bosch et al., 2017); however, effects on pups' survival, development and body composition were not reported in the same studies.

## **Conclusion**

In conclusion, this was one of the first studies to evaluate effects of loss of a mate on mothers in a biparental mammal. Effects of single motherhood on mothers' behavior, morphology, and physiology suggest that rearing offspring alone is energetically costly and can induce organ remodeling and changes in HPA activity, even when animals are housed under benign laboratory conditions with abundant food and water, mild temperature and humidity, and absence of predators. Although single mothers in the present study were able to produce a similar number and quality of offspring and showed no differences in behavioral indicators of emotionality compared to paired mothers, it is possible that more severe effects of being a single mother on the females, and/or effects of having a single mother on the offspring, could be revealed under more challenging or ecologically relevant conditions.

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## Figure Captions

Fig. 1. Experimental timeline. Adult females were weighed twice weekly from pair formation to parturition, and pups were checked daily for eye-opening. NB – Non-breeding females, PM – paired mothers, SM – single mothers, CORT – corticosterone.

Fig. 2. Towered cages used in the CLIMB condition. Food was located at the top of one tower, and a water bottle was at the top of the other tower.

Fig. 3. Estimated marginal means (EMM) and associated standard errors (SE) of number of fecal boli produced during A: 10-minute open-field tests and B: 6-minute tail-suspension tests. Statistical results shown are for pairwise comparisons among reproductive groups; neither the main effect of housing condition nor the housing condition  $\times$  reproductive group interaction was significant for either measure. Light gray, standard cages; dark gray, towered cages.

Fig. 4. EMM  $\pm$  SE baseline plasma corticosterone concentrations across the diel cycle in female California mice. A: CORT levels of all females housed in standard cages (CTRL) or in cages with towers requiring the mice to climb to obtain food and water (CLIMB), collapsed across reproductive conditions.

Fig. 5. EMM  $\pm$  SE plasma corticosterone concentrations at 12:00 h under baseline conditions and immediately after a 6-minute tail-suspension test. The tail-suspension test significantly increased plasma CORT. A: CORT levels in all females housed in standard cages (CTRL) or in

cages with towers requiring the mice to climb to obtain food and water (CLIMB), collapsed across reproductive conditions. B: CORT levels of single mothers (SM), paired mothers (PM), and non-breeding females (NB), collapsed across housing conditions.

Fig. 6. EMM  $\pm$  SE of mean lean mass and organ masses of female California mice. Ovary and triceps surae masses are means of the two paired organs. Statistical results shown are for pairwise comparisons among reproductive groups; neither the main effect of housing condition nor the housing condition  $\times$  reproductive group interaction was significant for any measure. Light gray, standard cages; dark gray, towered cages.

Fig. 7. EMM  $\pm$  SE of gastrointestinal masses of female California mice. Statistical results shown are for pairwise comparisons among reproductive groups; neither the main effect of housing condition nor the housing condition  $\times$  reproductive group interaction was significant for any measure. Light gray, standard cages; dark gray, towered cages.

Table 1. Results of Pearson correlations and paired t-tests comparing values for paired organ sizes. Positive t values indicate that left > right. Significant P values ( $P \leq 0.016$  when modified for Adaptive False Discovery Rate and Cohen's  $d > 0.5$  for pair-wise comparisons) are underlined.

Organ	Unit	Transform	N of paired observations	r of Pearson correlation	P of Pearson correlation	t of paired t-test	P of paired t-test	Cohen's d
Triceps surae	gram	none	80	0.86	<u>1.66E-24</u>	1.11	0.270	0.066
Ovary	gram	none	79	0.77	<u>5.77E-17</u>	0.03	0.979	0.002
Adrenal	gram	none	80	0.82	<u>1.85E-20</u>	5.82	1.22E-07	0.392
Kidney	gram	none	80	0.93	<u>1.44E-35</u>	-1.99	0.0501	0.085
Femur	gram	none	77	0.78	<u>6.66E-17</u>	-1.13	0.263	0.086
Femur	millimeter	none	77	0.62	<u>1.86E-09</u>	0.17	0.868	0.017
Foot	millimeter	none	76	0.52	<u>1.00E-06</u>	1.17	0.246	0.132

Table 2. Results of ANCOVAs comparing three reproductive groups (non-breeding females, paired mothers, single mothers) under two housing conditions (standard cages, towered cages) with significant group differences. Significant P values ( $P \leq 0.016$  when modified for Adaptive False Discovery Rate and  $\eta^2 > 0.06$ ) are underlined.

Measure	Unit	Transform	Repro. Group				Housing Condition				Repro. Group * Housing Condition			
			F	D.F.	P	Partial eta squared	F	D.F.	P	Partial eta squared	F	D.F.	P	Partial eta squared
Open field: number of fecal boli	number	none	4.50	2,80	<u>0.014</u>	0.101	0.62	1,80	0.433	0.008	0.26	2,80	0.769	0.007
Tail suspension: number of fecal boli	number	none	3.14	2,77	<u>0.049</u>	0.075	0.10	1,77	0.752	0.001	0.23	2,77	0.796	0.006
Heart mass	gram	none	3.99	2,72	<u>0.023</u>	0.100	0.07	1,72	0.790	0.001	0.46	2,72	0.635	0.013
Liver mass	gram	log <sub>10</sub>	8.55	2,72	<u>4.68E-04</u>	0.192	0.32	1,72	0.573	0.004	0.18	2,72	0.835	0.005
Triceps surae mass (mean of left and right)	gram	rank	5.25	2,69	<u>0.008</u>	0.132	0.12	1,69	0.732	0.002	0.30	2,69	0.745	0.008
Ovary mass (mean of left and right)	gram	**0.5	6.96	2,71	<u>0.002</u>	0.164	0.01	1,71	0.932	1.02E-04	0.39	2,71	0.681	0.011
Uterus mass	gram	log <sub>10</sub>	5.85	2,71	<u>0.004</u>	0.141	0.75	1,71	0.390	0.010	0.22	2,71	0.807	0.006
Stomach mass	gram	none	6.53	2,69	<u>0.003</u>	0.159	1.33	1,69	0.252	0.019	0.59	2,69	0.557	0.017
Caecum mass	gram	log <sub>10</sub>	5.78	2,69	<u>0.005</u>	0.144	0.26	1,69	0.610	0.004	1.47	2,69	0.236	0.041
Large intestine mass	gram	none	5.51	2,69	<u>0.006</u>	0.138	0.18	1,69	0.677	0.003	1.84	2,69	0.166	0.051
Small intestine mass	gram	log <sub>10</sub>	4.54	2,69	<u>0.014</u>	0.116	0.51	1,69	0.477	0.007	0.43	2,69	0.656	0.012

2843															
2844															
2845															
2846	Postpartum body mass 4	gram	none	3.20	2,78	<u>0.046</u>	0.076	1.20	1,78	0.276	0.015	0.98	2,78	0.378	0.025
2847															
2848	Postpartum lean mass														
2849	(mean of lean masses 1-4)	gram	**0.5	3.69	2,81	<u>0.029</u>	0.083	0.47	1,81	0.495	0.006	1.98	2,81	0.145	0.047
2850															
2851	Postpartum lean mass 2	gram	rank	11.20	2,80	<u>0.003</u>	0.134	0.96	1,80	0.763	0.001	0.36	2,80	0.345	0.026
2852															
2853	Postpartum lean mass 3	gram	**0.5	7.43	2,80	<u>0.001</u>	0.157	1.33	1,80	0.252	0.016	0.64	2,80	0.530	0.016
2854															
2855	Percent fat mass 2	<b>percent</b>	rank	4.48	2,80	<u>0.014</u>	0.101	0.01	1,80	0.947	5.60E-05	1.47	2,80	0.235	0.036
2856															
2857	Percent fat mass 3	<b>percent</b>	rank	3.64	2,79	<u>0.031</u>	0.084	0.09	1,79	0.771	0.001	2.25	2,79	0.112	0.054
2858															
2859	Percent lean mass 2	<b>percent</b>	rank	5.15	2,80	<u>0.008</u>	0.114	0.00	1,80	0.975	1.20E-05	1.45	2,80	0.242	0.035
2860															
2861	Percent lean mass 4	<b>percent</b>	rank	3.13	2,72	<u>0.0496</u>	0.080	0.12	1,72	0.725	0.002	2.39	2,72	0.099	0.062
2862															
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Table 3. Results of ANOVAs comparing pups reared by single mothers and paired mothers in the two housing conditions (standard cages, towered cages).

Measure	Unit	Transform	Repro. Group				Housing Condition				Repro. Group * Housing Condition			
			F	D.F.	P	Partial eta squared	F	D.F.	P	Partial eta squared	F	D.F.	P	Partial eta squared
Litter size at weaning	pup	none	1.41	1,55	0.239	0.025	0.12	1,55	0.732	0.002	0.01	1,55	0.937	1.14E-04
Pups' age of eye opening (eye opening of the first pup of the litter)	day	none	0.23	1,55	0.635	0.004	0.20	1,55	0.658	0.004	0.69	1,55	0.411	0.012
Pups' fat mass (mean of all pups in litter)	gram	log <sub>10</sub>	0.15	1,51	0.704	0.003	1.28	1,51	0.263	0.024	1.09	1,51	0.302	0.021
Pups' lean mass (mean of all pups in litter)	gram	none	0.56	1,51	0.458	0.011	1.94	1,51	0.169	0.037	0.25	1,51	0.618	0.005
Pups' percent fat mass (mean of all pups in litter)	percent	log <sub>10</sub>	0.03	1,51	0.859	0.001	0.23	1,51	0.637	0.004	0.86	1,51	0.359	0.017
Pups' percent lean mass (mean of all pups in litter)	percent	rank	0.01	1,51	0.923	1.85E-04	0.94	1,51	0.336	0.018	0.46	1,51	0.503	0.009
Pups' body mass (mean of all pups in litter)	gram	none	1.14	1,53	0.290	0.021	3.11	1,53	0.084	0.055	0.28	1,53	0.600	0.005
Pups' body mass (total body mass of litter)	gram	none	2.61	1,53	0.112	0.047	3.43	1,53	0.070	0.061	0.02	1,53	0.884	4.02E-04

Supplementary Table 1. Results of ANCOVAs comparing the three reproductive groups (non-breeding females, paired mothers, single mothers) in the two housing conditions (standard cages, towered cages). Significant P values ( $P \leq 0.016$  when modified for Adaptive False Discovery Rate and  $\eta^2 > 0.06$ ) are underlined.

Measure	Unit	Transform	Repro. Group				Housing Condition				Repro. Group * Housing Condition			
			F	D.F.	P	Partial eta squared	F	D.F.	P	Partial eta squared	F	D.F.	P	Partial eta squared
Novel object: latency to approach object	second	rank	0.68	2,81	0.510	0.016	0.21	1,81	0.648	0.003	0.12	2,81	0.890	0.003
Novel object: bouts of approaching object	number	**0.5	0.04	2,81	0.966	0.001	1.08	1,81	0.303	0.013	0.54	2,81	0.584	0.013
Novel object: duration of immobility	second	log <sub>10</sub>	1.20	2,81	0.312	0.054	0.20	1,81	0.658	0.005	0.51	2,81	0.603	0.024
Novel object: durations of sniffing and touching object	second	log <sub>10</sub>	0.85	2,81	0.430	0.021	0.32	1,81	0.574	0.004	1.15	2,81	0.323	0.028
Saccharine preference (proportion of total fluid consumption)	proportion	none	0.39	2,80	0.678	0.010	4.87	1,80	<u>0.030</u>	0.057	1.44	2,80	0.243	0.035
Open field: number of crossing bouts	number	log <sub>10</sub>	0.86	2,74	0.427	0.047	0.15	1,74	0.702	0.005	0.27	2,74	0.766	0.001
Open field: duration in outer region	second	rank	0.02	2,73	0.978	0.001	0.04	1,73	0.845	0.001	1.50	2,73	0.230	0.039
Open field: duration in inner region	second	**0.5	0.27	2,74	0.762	0.007	0.29	1,74	0.592	0.004	1.52	2,74	0.225	0.039
Open field: distance traveled in outer region	meter	**0.5	2.62	2,74	0.079	0.066	0.18	1,74	0.669	0.002	0.18	2,74	0.836	0.005



2969															
2970															
2971															
2972	Open field:														
2973	distance traveled in inner	meter	**0.5	0.27	2,68	0.767	0.007	0.23	1,68	0.637	0.003	0.13	2,68	0.882	0.004
2974	region														
2975	Open field:														
2976	total distance traveled in	meter	none	1.32	2,71	0.274	0.036	0.39	1,71	0.535	0.005	0.01	2,71	0.988	3.50E-04
2977	inner and outer regions														
2978	Open field:														
2979	number of fecal boli	number	none	4.50	2,80	<u>0.014</u>	0.101	0.62	1,80	0.433	0.008	0.26	2,80	0.769	0.007
2980															
2981	Open field:														
2982	number of urine pools	number	**0.5	0.41	2,80	0.665	0.010	0.58	1,80	0.447	0.007	0.20	2,80	0.822	0.005
2983															
2984	Tail suspension:														
2985	duration of mobility	minute	none	0.51	2,81	0.602	0.013	0.07	1,81	0.793	0.001	0.14	2,81	0.869	0.004
2986															
2987	Tail suspension:														
2988	number of fecal boli	number	none	3.14	2,77	<u>0.049</u>	0.075	0.10	1,77	0.752	0.001	0.23	2,77	0.796	0.006
2989															
2990	Heart mass	gram	none	3.99	2,72	<u>0.023</u>	0.100	0.07	1,72	0.790	0.001	0.46	2,72	0.635	0.013
2991															
2992	Liver mass	gram	log <sub>10</sub>	8.55	2,72	<u>4.68E-04</u>	0.192	0.32	1,72	0.573	0.004	0.18	2,72	0.835	0.005
2993															
2994	Spleen mass	gram	log <sub>10</sub>	0.22	2,72	1.526	0.041	0.73	1,72	0.125	0.002	0.85	2,72	0.166	0.005
2995															
2996	Triceps surae mass	gram	rank	5.25	2,69	<u>0.008</u>	0.132	0.12	1,69	0.732	0.002	0.30	2,69	0.745	0.008
2997	(mean of left and right)														
2998	Ovary mass														
2999	(mean of left and right)	gram	**0.5	6.96	2,71	<u>0.002</u>	0.164	0.01	1,71	0.932	1.02E-04	0.39	2,71	0.681	0.011
3000															
3001	Uterus mass	gram	log <sub>10</sub>	5.85	2,71	<u>0.004</u>	0.141	0.75	1,71	0.390	0.010	0.22	2,71	0.807	0.006
3002															
3003	Adrenal mass														
3004	(mean of left and right)	gram	rank	1.21	2,72	0.306	0.032	0.05	1,72	0.830	0.001	0.29	2,72	0.753	0.008
3005															
3006															
3007															
3008															
3009															
3010															

3011															
3012															
3013															
3014	Kidney mass	gram	none	0.28	2,72	0.759	0.008	0.00	1,72	0.960	3.50E-05	0.53	2,72	0.590	0.015
3015	(mean of left and right)														
3016															
3017	Stomach mass	gram	none	6.53	2,69	<u>0.003</u>	0.159	1.33	1,69	0.252	0.019	0.59	2,69	0.557	0.017
3018															
3019	Caecum mass	gram	log <sub>10</sub>	5.78	2,69	<u>0.005</u>	0.144	0.26	1,69	0.610	0.004	1.47	2,69	0.236	0.041
3020															
3021	Large intestine mass	gram	none	5.51	2,69	<u>0.006</u>	0.138	0.18	1,69	0.677	0.003	1.84	2,69	0.166	0.051
3022															
3023	Small intestine mass	gram	log <sub>10</sub>	4.54	2,69	<u>0.014</u>	0.116	0.51	1,69	0.477	0.007	0.43	2,69	0.656	0.012
3024															
3025	Femur mass	gram	none	0.00	2,67	0.999	2.60E-05	0.01	1,67	0.928	1.22E-04	0.25	2,67	0.783	0.007
3026	(mean of left and right)														
3027															
3028	Femur length	millimeter	none	0.96	2,70	0.386	0.027	0.95	1,70	0.333	0.013	0.17	2,70	0.844	0.005
3029	(mean of left and right)														
3030															
3031	Foot length	millimeter	none	0.49	2,69	0.617	0.014	0.38	1,69	0.538	0.006	0.15	2,69	0.862	0.004
3032	(mean of left and right)														
3033															
3034	Tail length	millimeter	none	0.40	2,70	0.671	0.011	1.93	1,70	0.169	0.027	0.79	2,70	0.460	0.022
3035															
3036	Postpartum body mass	gram	none	0.79	2,80	0.459	0.019	0.54	1,80	0.464	0.007	1.40	2,80	0.253	0.034
3037	(mean of body masses 1-5)														
3038															
3039	Postpartum body mass 1	gram	log <sub>10</sub>	0.60	2,76	0.554	0.015	0.22	1,76	0.645	0.003	1.29	2,76	0.282	0.033
3040															
3041	Postpartum body mass 2	gram	none	0.22	2,79	0.801	0.006	0.10	1,79	0.757	0.001	1.63	2,79	0.203	0.04
3042															
3043	Postpartum body mass 3	gram	none	2.18	2,81	0.120	0.051	1.55	1,81	0.216	0.019	1.67	2,81	0.195	0.040
3044															
3045	Postpartum body mass 4	gram	none	3.20	2,78	<u>0.046</u>	0.076	1.20	1,78	0.276	0.015	0.98	2,78	0.378	0.025
3046															
3047	Postpartum body mass 5	gram	none	2.09	2,68	0.132	0.058	0.88	1,68	0.352	0.013	0.71	2,68	0.494	0.021
3048															
3049															
3050															
3051															
3052															

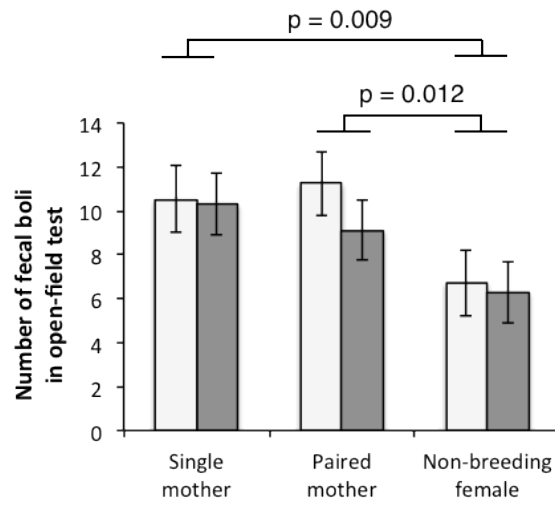
3053															
3054															
3055															
3056	Postpartum fat mass	gram	rank	1.06	2,81	0.353	0.025	0.09	1,81	0.766	0.001	2.20	2,81	0.117	0.052
3057	(mean of fat masses 1-4)														
3058															
3059	Postpartum fat mass 1	gram	rank	1.38	2,76	0.258	0.035	0.10	1,76	0.749	0.001	0.65	2,76	0.526	0.017
3060															
3061	Postpartum fat mass 2	gram	rank	2.82	2,80	0.066	0.066	0.00	1,80	0.996	3.39E-07	2.22	2,80	0.115	0.053
3062															
3063	Postpartum fat mass 3	gram	rank	1.89	2,80	0.158	0.045	0.03	1,80	0.874	3.20E-04	2.28	2,80	0.109	0.054
3064															
3065	Postpartum fat mass 4	gram	rank	1.74	2,73	0.183	0.046	0.05	1,73	0.830	0.001	3.02	2,73	0.055	0.077
3066															
3067	Postpartum lean mass	gram	**0.5	3.69	2,81	<u>0.029</u>	0.083	0.47	1,81	0.495	0.006	1.98	2,81	0.145	0.047
3068	(mean of lean masses 1-4)														
3069															
3070	Postpartum lean mass 1	gram	rank	2.52	2,76	0.087	0.062	0.08	1,76	0.772	0.001	1.16	2,76	0.321	0.029
3071															
3072	Postpartum lean mass 2	gram	rank	11.20	2,80	<u>0.003</u>	0.134	0.96	1,80	0.763	0.001	0.36	2,80	0.345	0.026
3073															
3074	Postpartum lean mass 3	gram	**0.5	7.43	2,80	<u>0.001</u>	0.157	1.33	1,80	0.252	0.016	0.64	2,80	0.530	0.016
3075															
3076	Postpartum lean mass 4	gram	**2	2.31	2,73	0.106	0.06	0.01	1,73	0.912	1.69E-04	1.37	2,73	0.260	0.036
3077															
3078	Percent fat mass	<b>percent</b>	rank	2.96	2,81	0.058	0.058	0.39	1,81	0.532	0.532	1.71	2,81	0.187	0.187
3079	(mean of percent fat masses 1-4)														
3080															
3081	Percent fat mass 1	<b>percent</b>	rank	1.66	2,76	0.197	0.042	0.06	1,76	0.809	0.001	0.48	2,76	0.621	0.012
3082															
3083	Percent fat mass 2	<b>percent</b>	rank	4.48	2,80	<u>0.014</u>	0.101	0.01	1,80	0.947	5.60E-05	1.47	2,80	0.235	0.036
3084															
3085	Percent fat mass 3	<b>percent</b>	rank	3.64	2,79	<u>0.031</u>	0.084	0.09	1,79	0.771	0.001	2.25	2,79	0.112	0.054
3086															
3087	Percent fat mass 4	<b>percent</b>	rank	1.35	2,72	0.265	0.036	0.25	1,72	0.620	0.003	2.11	2,72	0.129	0.055
3088															
3089	Percent lean mass	<b>percent</b>	rank	1.80	2,81	0.171	0.043	0.05	1,81	0.831	0.001	1.71	2,81	0.187	0.041
3090	(mean of percent lean masses 1-4)														
3091															
3092															
3093															
3094															

3095															
3096															
3097															
3098	Percent lean mass 1	<b>percent</b>	rank	0.22	2,75	0.804	0.006	0.01	1,75	0.918	1.41E-04	0.46	2,75	0.635	0.012
3099															
3100	Percent lean mass 2	<b>percent</b>	rank	5.15	2,80	<u>0.008</u>	0.114	0.00	1,80	0.975	1.20E-05	1.45	2,80	0.242	0.035
3101															
3102	Percent lean mass 3	<b>percent</b>	rank	2.79	2,79	0.068	0.066	0.12	1,79	0.733	0.001	1.77	2,79	0.177	0.043
3103															
3104	Percent lean mass 4	<b>percent</b>	rank	3.13	2,72	<u>0.0496</u>	0.0800	0.12	2,72	0.725	0.002	2.39	2,72	0.099	0.062
3105	<hr/>														
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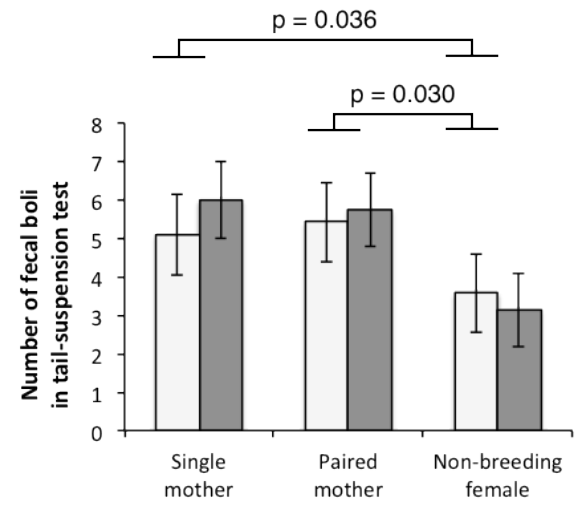
Day of Expt.	Test Day	Time	Procedure(s)
~ -59			Vasectomize NB males
~ -45			Pair formation; move half of pairs to climbing cages
~ (-29)-(-24)			Vasectomize SM & PM males; move all pairs to divided cages for 1 week
0			Birth
1		09:00-10:00 h	Remove SM fathers
3	1	14:00-16:00 h	Body composition
6	2	04:00 h	Blood sample (basal CORT)
9	3	20:00 h	Blood sample (basal CORT)
12	4	14:00-16:00 h	Novel-object test; body composition
14	5	20:00-22:00 h	Behavioral observation
15	6	19:00-23:00 h	Saccharine preference test
17	7	14:00-16:00 h	Behavioral observation
18	8	14:00-16:00 h	Open-field test; body composition
20	9	8:00-10:00 h	Behavioral observation
21	10	12:00 h	Blood sample (basal CORT)
23	11	2:00-4:00 h	Behavioral observation
24	12	11:30-12:35 h	Tail-suspension test; blood collection (post-stress CORT), body mass
26	13	14:00-16:00 h	Body composition
27	14	14:00-16:00 h	Dissection

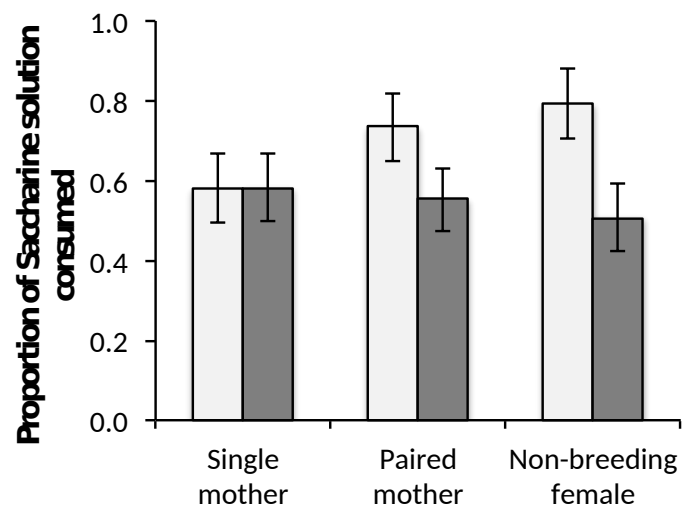


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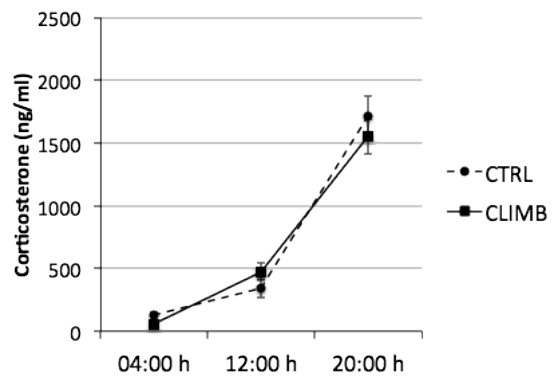
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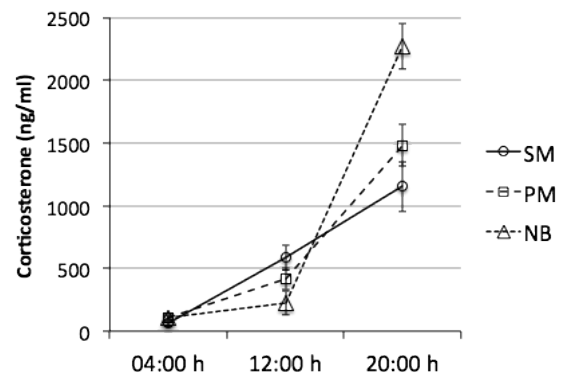




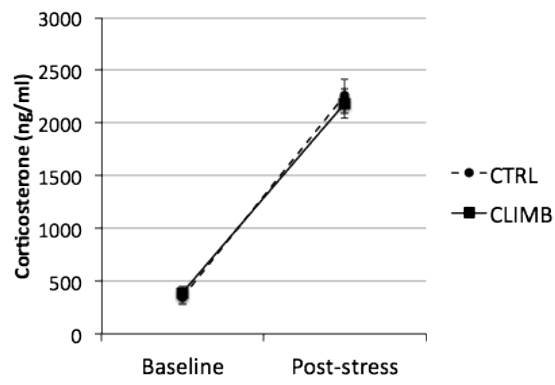
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